AD_____

Award Number: DAMD17-97-1-7194

TITLE: Production and Characterization of High Affinity Single-chain Antibody Fragments (scFvst) That Inhibit Breast Cancer Metastasis

PRINCIPAL INVESTIGATOR: James D. Marks, M.D., Ph.D.
David Powers, Ph.D.

CONTRACTING ORGANIZATION: University of California, San Francisco
San Francisco, California 94143-0962

REPORT DATE: October 2000

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 2000	Final (30 Sep 97 - 29 Sep 00)

4. TITLE AND SUBTITLE
Production and Characterization of High Affinity Single-Chain Antibody Fragments (scFvst) that Inhibit Breast Cancer Metastasis

5. FUNDING NUMBERS
DAMD17-97-1-7194

6. AUTHOR(S)
James D. Marks, M.D., Ph.D.
David Powers, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of California , San Francisco
San Francisco, California 94143-0962

e-mail:
marksj@anesthesia.ucsf.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

We have generated high affinity, specific single chain antibodies that recognize two key components of the metastatic phenotype: urokinase-type plasminogen activator (uPA) and its cell surface receptor (uPA-R). A total of six different scFv antibodies were found for each of the two antigens. Competition ELISA assays show that 5 of the 6 anti-uPA-R scFv antibodies compete with uPA for binding to the receptor and therefore are candidates for scFv which may inhibit metastasis by disrupting the uPA:uPA-R interaction. We have also developed the technology to convert a scFv selected from a phage display library, which due to its small size has an extremely short serum half life in mouse, into a longer lived format to allow for *in vivo* anti-metastasis assays. We have accomplished this by creating plasmid vectors that fuse the scFv to the human IgG1 Fc domain. The resulting scFv-Fc fusion is expressed to high levels in yeast cells, retains the affinity and specificity of the parent scFv, and has a serum half life in mice of 93 hours, similar to a IgG molecule.

14. SUBJECT TERMS
Breast Cancer, scFv , phage antibodies, metastasis, urokinse plaminogen activator

15. NUMBER OF PAGES
27

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

__✓__ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

__✓__ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

__✓__ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

__✓__ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

__✓__ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

__✓__ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

PI Signature

8/29/01
Date

Table of Contents

5. Introduction

5.1 Overview and goals of the project

For the breast cancer patient, it is metastasis which all too often ultimately proves fatal. Metastasis is a complex, multistep process by which tumor cells invade surrounding host tissue, enter the bloodstream, travel through the circulatory system to a distant site, and exit from the blood vessel to establish a metastatic colony. The metastatic process involves multiple tumor:host interactions, such as proteolysis of the extracellular matrix and attachment to target tissues; as well as autocrine responses of the tumor cells to motility factors, and angiogenesis-inducing factors. Each step in the metastatic process represents a possible point of therapeutic intervention; and since most of these steps are mediated by interactions involving extracellular molecules, they are in principle amenable to inhibition by engineered antibodies. We proposed to create single-chain human Fv antibody fragments (scFvs) to proteins involved in metastasis by selection from phage display libraries. These scFvs would be used to dissect the contribution of different molecular interactions to the overall metastatic process using various *in vitro* and *in vivo* assays, and might eventually serve as the basis for anti-metastasis therapeutic agents.

As a starting point toward this larger goal, we created high-affinity scFv molecules directed towards the cell-surface receptor of urokinase-type plasminogen activator: uPA-R. Urokinase (uPA) and its receptor (uPA-R) are key components of the metastatic machinery. Urokinase (uPA) is a 52,000 dalton serine protease which is overexpressed in many breast tumors where it is found in a membrane-bound form through interaction with uPA-R. Substantial evidence exists for a key role of receptor-bound uPA activity in metastasis via proteolysis of basement membranes by activating various zymogens to active proteases. In breast cancer, uPA levels are a prognostic factor for relapse-free and overall- survival. Inhibition of receptor-bound uPA activity, either by blocking uPA protease activity itself or by blocking uPA binding to its receptor can inhibit metastasis in a number of *in vitro* and *in vivo* metastasis model systems. To study the role of the uPA:uPA-R interaction in metastasis, we proposed to generate high-affinity scFv species to the uPA receptor which compete with uPA for binding. These molecules were to be tested for antimetastasis activity using an *in vitro* basement membrane degradation assay and a sensitive *in vivo* metastasis assay using breast tumor xenografts in nude mice.

A key subgoal of this research is to develop the technology to create single chain antibody variants with proper pharmacokinetic properties such that they can be used for *in vitro and in vivo* assays. This is important not only for the purpose of this anti-metastasis research, but as a key technology to support other research efforts in the laboratory of Dr. James Marks to develop and test antibodies that attack breast cancer through growth factor receptors or molecules involved in angiogenesis. This report will detail results in the selection of single chain antibody fragments (scFv) from phage display libraries toward urokinse and its receptor, and the development of technology to modify these scFv such that they can be tested for anti-metastasis activity *in vivo.*

The proposed technical objectives in the statement of work were:

Technical objective 1: Generate and characterize human scFv antibodies to urokinase-type plaminogen activator (uPA)

Technical objective 2: Generate and characterize human scFv antibodies to urokinase-type plaminogen activator (uPAR)

Technical objective 3: Test scFv for in vitro and in vivo inhibition of metastasis

6. Body of report

6.1 Selections on urokinase (uPA) and urokinase receptor (uPA-R) (technical objectives 1 &2)

To identify high affinity single chain antibodies to urokinase (uPA) and its receptor (uPA-R), selections were performed using a large (7×10^9 clones) non-immune human scFv antibody library expressed on the surface of bacteriophage (*1*). Urokinase was obtained commercially (ICN Biomedical, Aurora, Ohio); soluble urokinase receptor was a gift of Dr. Mark Shuman, University of California at San Francisco. The antigens were coated on the surface of immunotubes (Nunc) at 50 ug/ml in 100 mM carbonate buffer pH 6.2. The tubes containing the immobilized antigens were blocked with 4% powdered milk in phosphate buffered saline (MPBS), then 5×10^{12} phagemid particles were added and allowed to bind for 1 hour. Non-binding phage were washed away (20 washes with PBS + .05 % Tween 20 (TPBS) and 20 washes with phosphate buffered saline (PBS)), then binding phage were eluted with 1.0 ml of 100 mM triethylamine, and immediately neutralized by the addition of 0.5 ml 1 M Tris pH 7.5. The eluted phage were amplified by infection of *E. coli* TG1 cells, and used for the next round of selection. A total of 3 rounds of selection were performed for each of the two antigens.

Selections were monitored by determining the titer of eluted phage at each round of selection; in a typical successful selection the output phage titer will increase in subsequent rounds as phage bearing a scFv that binds to the target antigen are enriched in the phagemid population. Results are shown in Table 1 (appendix 1). For both antigens, starting titers of ~10^4-10^5 increased two logs or more to ~10^7 in the final round, indicating successful enrichment of phage displaying scFv specific for the target antigen. At each round of selection, 96 separate clones were picked and grown in a 96 well microtiter plate for analysis.

6.2 Characterization of selected clones

6.2.1 ELISA results

To determine which of the selected phagemid clones were specific for the target antigens, small scale expression cultures of the clones were grown to express soluble scFv fragments for analysis. The library clones are in vector pHEN1 which fuses the scFv clone to the minor phage coat protein p3 through an amber stop codon (UAG). In supressor strains of *E. coli* such as TG1 the amber codon is supressed, resulting in incorporation of an amino acid at the amber codon and fusion of the scFv to the p3 protein. However the supression is not 100% efficient; more than half of the scFv produced terminate at the amber codon and are released in a soluble form to the culture medium. Thus small scale cultures of scFv phagemid clones in *E. coli* TG1 strain produce sufficient quantities of soluble scFv for ELISA analysis. In addition, the scFv produced from vector pHEN-1 are fused to a myc epitope tag, allowing for detection with anti-myc antibodies.

Expression of scFv for ELISA analysis was achieved as follows: cultures of 96 clones apiece from the first, second, and third rounds of selection of the two antigens were grown by replicating plating from the master plates into fresh 96 well microtiter plates containing 150 ul per well of 2xYT media supplemented with ampicillin and 0.1% glucose. The expression plates were grown at 37° C for 3-4 hours with 200 rpm shaking, until the OD_{550} of the bacteria was approximately 1.0. Then 50 ul of a 4 mM IPTG solution was added to induce scFv synthesis, and the plates incubated for an additional 8-12 hours at 25° C with shaking. Finally the cells were removed by centrifugation, and the scFv-containing supernatants from each well were removed and stored at 4°C for ELISA and other analyses.

ELISAS were performed as follows: 96-well microtiter plates were coated with antigens at 10 ug/ml in 100 mM carbonate buffer pH 6.2 overnight. The next day, the plates were blocked by the addition of 200 ul/well of MPBS (PBS plus 4% powdered milk) for 1 hour. The plates were washed with PBS, then 50 ul of the *E. coli* expression supernatants were added per well and allowed to bind for 1.5 hours at room temperature. Finally after washing, bound scFv were detected with the anti-myc monoclonal antibody 9E10 (Santa Cruz Biochemicals) and an anti-mouse IgG HRP (horse radish peroxidase) conjugate and developed with the peroxidase

substrate 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS, Sigma). Positive clones were defined as those which had an A_{405} reading of at least twice background.

ELISA results are shown in Table 2 (appendix 1). For both antigens, the percentage of positive clones from the first to second and from second to third rounds, indicating enrichment of clones bearing scFv specific for the target antigen as desired. No specific binders were detected for 96 random clones from the unselected library for either antigen (data not shown). A total of 37 total clones were detected for urokinase, and 46 for the urokinase receptor.

6.2.2 Sequence Analysis

The positive clones for uPA and uPA-R were analyzed by PCR-BstN1 fingerprinting (2) and finally clones having unique PCR patterns were sequenced by dideoxy chain termination on a Licor model 400L DNA sequencer (data not shown). Sequence analysis was necessary both to determine the number of unique clones for further characterization as well as to design PCR primers for further subcloning manipulations of these clones (see section 2.3) A total of 6 unique antigen-positive clones were detected for uroknase receptor: the clones identified that bound to uPA-R were denoted G12, A4, C2, F5, B5, and A9. Sequence analysis showed that the selection also resulted in the generation of six unique scFv antibodies to urokinase (uPA).

6.2.3 Competition ELISA analysis of anti-uPA-R scFv antibodies

scFv antibodies selected on the urokinase plasminogen activator receptor uPA-R were next assayed by a competition ELISA in which we sought to determine if the epitope recognized by the scFv overlapped with the uPA binding site on the receptor. For these studies, the scFv was subcloned into the vector pUC119mychis as Nco1-Not1 fragments. The vector affixes a C-terminal hexahistidine tag at the scFv C-terminus, allowing subsequent purification by immobilized metal affinity chromatography. ScFv was expressed from 250 ml cultures of E. coli TG1 and scFv purified by immobilized metal affinity chromaotgraphy. To determine if the purified scFv blocked binding of uPA to uPA-R, an ELISA was performed in which uPA-R was bound to an immunoplate and scFv binding to the uPA-R were detected by a sandwich assay as described in section 6.2.1. A second set of parallel ELISA reactions were done in which the scFv binding was done in the presence of 5 ug uPA in 50 ul of PBS to compete with the scFv for binding to the uPA-R. Results were calculated as a percentage of maximum signal seen in the presence of uPA and are shown in Table 3. The results show that only one out of the six anti-uPA-R scFv recognizes an epitope that does not overlap with the uPA binding site, as this scFv bind to uPA-R as well in the presence of uPA as by itself (B5). For the other five scFv, significant reduction of maximal signal (30-50%) in the presence of uPA is seen, indicating that the epitopes recognized by these clones at least partially overlap with the uPA binding site on uPA-R. These 5 clones are candidates for antibody scFv fragments that may inhibit metastasis by inhibition of the uPA:uPA-R interaction.

6.3 Construction of scFv derivatives for in vivo studies

Before the anti-uPA receptor antibiodies could be tested for anti-metastasis activity *in vivo*, they needed to be converted to a format that would preserve the affinity and specificity of the parent scFv, whilst giving the scFv the long serum half life of an IgG species. This was done by developing the technology to express a genetic fusion of a scFv with the human Fc antibody domains as detailed in the following section. The basic design of this dimeric scFv-Fc fusion is shown schematically in Figure 1 in Powers et al., appendix 2. The development of this technology, in addition to its use to further the metastasis studies outlined, is also finding a key role in the laboratory of Dr. Marks for *in vivo* testing of antibodies to growth factors and angiogenesis factors which may be targets for antibody-based breast cancer therapeutics.

6.3.1 Rationale and introduction

The modular nature of antibody molecules allows an almost unlimited number of domain rearrangements. Antibody engineering allows the researcher to design and use a

variety of binding domains, effector domains, as well as non-antibody fusion partners (3). One of the more useful antibody fragments is the single-chain Fv, or scFv (4), in which the antibody V_H and V_L domains are joined by a short peptide linker. This monovalent, minimal binding fragment is favored for antibody phage display techniques and phage antibody library construction (2, 5).

Once a scFv has been isolated from a phage display library, the binding domain can be characterized as to affinity, epitope and biological activity, as well as subjected to further affinity maturation. The scFv can be used as is, or engineered into other forms (Fv, Fab, Fab2', IgG, or fused to other proteins).

For some applications the scFv itself is the desired format. The small size of the scFv and its rapid clearance from the blood makes it the molecule of choice for tumor targeting approaches (6, 7). The single-chain nature of the scFv is best-suited for intracellular immunization (intrabody) studies (8). However for other uses, it would be desirable to transfer the antigen-binding properties of the scFv into a full length IgG, to take advantage of avidity effects, effector functions, and the prolonged serum half life of an immunoglobulin. Increasing the serum half life of scFv is particularly important for demonstrating in vivo antigen neutralization since the $t_{1/2}\beta$ of scFv is only 2.5 hours in mice (9).

One approach is to directly engineer a phage displayed scFv into a full length IgG and express it in mammalian cells (10). However this can be a laborious, and the yields from tissue culture can be modest. An alternate method would be to engineer the scFv into a more 'IgG-like' structure. We have constructed a vector to rapidly express an scFv-Fc fusion (wherein the scFv is fused to the hinge, C_H2, and C_H3 domains of human IgG1, see Figure 1, appendix 2) in the methylotrophic yeast *Pichia pastoris*. The scFv-Fc fusion can easily be constructed in a single cloning step from a scFv and is secreted at high levels as a glycosylated dimer from *Pichia* cultures. The scFv-Fc fusion retains the affinity and specificity of the parent scFv, combined with the bivalency, prolonged serum half-life, and Fc-mediated effector function (ADCC) of an IgG.

6.3.2 Construction of vector pPIgG-1

We have constructed a plasmid for the expression of single-chain antibody Fv domains (scFv) fused to the human IgG1 Fc domain to make a bivalent, ~106,000 molecular weight scFv-Fc fusion (Figure 2, appendix 2). Plasmid pPIgG1 for the expression of the scFv-Fc fusions (comprising the scFv followed by the hinge, C_H2 and C_H3 domains of human IgG1) is based on the *Pichia pastoris* expression plasmid pPICZαA (Invitrogen). This plasmid uses the alcohol oxidase promoter (AOX1) for high level expression of heterologous proteins, which are secreted to the media under direction of the yeast α-factor signal sequence. The human hinge, C_H2, and C_H3 domains of IgG1 were cloned into the *Not*I and *Xba*I sites of pPICZαA, creating plasmid pPIgG1. The presence of a stop codon at the end of C_H3 results in the myc epitope tag and $(His)_6$ sequences from pPICzαA not being expressed in the fusion protein.

scFv genes to be expressed as scFv-Fc fusions in pPIgG1 are amplified by PCR and subcloned into pPIgG1 using the 5' *Xho*I site in the alpha factor signal sequence and the *Not*I site at the end of the multiple cloning site. The 5' PCR primer, in addition to appending a dangling *Xho*I site, also recreates the amino acid sequence of the alpha amylase signal from the *Xho*I site to the end of the signal peptide, positioning the 5' end of the scFv flush with the end of the signal. The 3' PCR primer can be either a sequence-specific primer which appends a *Not*I site onto the end of the scFv gene, or a universal downstream primer for vectors such as pHEN1 which already have a *Not*I site positioned at the end of the scFv gene. The Fc domain adds an additional 26,300 in molecular weight to the scFv polypeptide chain, for a calculated molecular weight of ~53,000 for a typical scFv-Fc polypeptide monomer. As the selections for anti-uPA and anti-uPA-R single chain antibodies were ongoing at the time of the pPIgG-1 vector development, we chose two scFv as models to test the scFv-fusion vector's performance: C25,

which recognizes the botulinum neurotoxin serotype A binding domain Hc (*11*), and C6.5, which recognizes the ErbB-2 protein (*12*). The constructs were electroporated into Pichia patoris strain GS115 for expression.

6.3.3 Expression and characterization of scFv-Fc fusions

Small scale expression and screening of Pichia transformants

Since expression from integrated pPICZαA-derived plasmids can vary depending on site of integration and copy number, multiple transformants were screened for expression level. Six clones apiece of the pPIgG1-C25 and pPIgG-C6.5 transformants (along with pPICZαA control) were grown in 10 mls of BMMY media at 30° C for 72 hours as described in Materials and Methods. Results for four clones of each scFv-Fc fusion are shown in Figure 3, appendix 2. By silver stain of the media samples (Panel A) under reducing conditions, a new band appears in the media that is not in the vector control, with an apparent molecular weight of ~70,000. Close inspection shows that there is actually a dimer of new material at this molecular weight. Western blotting analysis (panel B) shows that these bands react with an anti-human Fc specific antibody. No anti-Fc reactive material was seen in control cells transfected with the control plasmid. Under non-reducing conditions, this material migrates in SDS-PAGE with approximately twice the apparent molecular weight, indicating that the scFv-Fc fusion secreted to the media is primarily in the form of a disulfide-linked dimer (not shown). Expression of the C6.5-Fc clones is somewhat less than for the C25-Fc clones. Expression was first observed at 24 hours with best expression levels seen at 72 hours (not shown). Two well-expressing clones were chosen for large scale expression and analysis.

Large scale expression and purification of scFv-Fc fusions

One liter of culture media was use used to generate pure C25-Fc and C6.5-Fc fusions for characterization. Briefly, the purification employed was ammonium sulfate precipitation of the proteins, followed by dialysis, Protein G chromatography, and gel filtration. Figure 4, appendix 2, panel A shows fractions from a typical purification. After ammonium sulfate precipitation, the fusion protein band is clearly visible, appearing as a dimer at ~70,000 daltons (lanes 1 and 4). After Protein G chromatography (lanes 2 and 5) the material is substantially pure, with a number of smaller bands copurifying which represent breakdown products which also bind to Protein G and react with anti-Fc antibodies (not shown). These breakdown species are removed by gel filtration on S200 (lanes 3 and 6). Under non-reducing conditions, the material migrates with approximately twice the molecular weight, indicating that the purified scFv-Fc fusions are disulfide-linked dimers (Figure 4B, appendix 2). The yield after purification was ~2 mg per liter of culture for C25-Fc and ~300 ug per liter of culture for the C6.5-Fc fusion.

The final purified material migrates in reducing SDS-PAGE as a tight dimer with an apparent molecular weight of ~70,000 daltons. This is substantially larger than the predicted molecular weights of 53,000 daltons for the reduced C25-Fc and C6.5-Fc fusion monomers. The discrepancy in apparent molecular weight and the heterogeneity observed could be due to glycosylation and/or differential processing of the alpha amylase signal peptide. To explore these possibilities, the purified fusions were subjected to N-terminal sequencing, de-glycosylation analysis with PNGase F, and mass spectrometry analysis.

N-terminal analysis results indicated that both the upper and lower bands of both of the fusions began with the amino acid sequence EAEA..., indicating that the signal peptide cleavage had occured after the Kex2 site in the alpha amylase leader, but that Ste13 cleavage had not occured.

The samples were further analyzed by deglycosylation with PNGase F (Figure 4C, appendix 2). After deglycosylation of both the fusion proteins, the doublet collapsed to a single band, indicating that the observed heterogeneity is due to difference in N-linked glycosylation.

Since SDS-PAGE can give spurious estimates of molecular weights, samples of the C25-Fc and C6.5-Fc were subjected to mass spectometry analysis. The molecular weights for the species were ~110,000 for both the C6.5-Fc and C25-Fc, in agreement with the predicted molecular weight from DNA sequence.

Pharmacokinetics

Single-chain Fv antibodies are rapidly cleared from the bloodstream in mouse models, with typical $t_{1/2}$ of the beta clearance of approximately 2.5 hours [Huston, 1996 #19]. This rapid clearance limits the usefulness of scFv in many potential animal models for efficacy where a longer serum half life is required. To test the pharmacokinetics of scFv-Fc fusions in mice, the C25-Fc fusion protein was radiolabelled and administered to scid mice in 20 ug doses, both intravenously and intraperitoneally. Figure 5, appendix 2 shows that the C25-Fc fusion had dramatically prolonged serum perseverence, with a $t_{1/2}$ for the beta phase 93 hours. The increased retention of the scFv-Fc fusions can be attributed to the increased size of the scFv-Fc homodimer which places it well above the renal threshhold for clearance.

6.3.4 Utility of scFv fusion antibodies

Single chain Fv antibody fragments (scFv) are useful molecules for the design and construction of phage display libraries. This format is easy to construct in a library format and allows for monovalent display and selection in a single gene format. For these reasons the scFv is the format of choice for phage display antibody efforts.

Once an initial scFv is selected, however, this structure has a number of potential limitations for various types of characterization. The monovalent nature of the scFv makes it unable to partake in the additional binding interactions due to avidity as seen in a bivalent antibody. The limitation of off rate imposed by an scFv can limit the effectiveness of these molecules in many immunochemical applications such as FACS, ELISA, etc. The commonly utilized epitope tags for detection (Myc, E, FLAG) can be subject to proteolytic removal either during or after purification (Marks *et al.* unpublished observations). Also, in-vivo characterization of scFv's in animal models is limited by their rapid clearance from the bloodstream due to their small size (*9*).

For many applications of characterizing a scFv it would be desirable to have a more "IgG-like" structure that combines the affinity and specificity of the scFv with the bivalency, pharmacokinetics, and effector functions of a complete immunoglobulin. One way is to clone the V_H and V_L genes from the scFv and reclone them into a full-length IgG expressing vector. However this can be laborious and time-consuming, especially if many such constructs have to be made and tested.

We have designed an intermediate construct, an scFv-Fc fusion in which the scFv is fused to the hinge, C_H2, and C_H3 domains of human IgG1, and shown that the fusion can be expressed and secreted as a glycosylated, disulfide-linked dimer in *Pichia pastoris*. The scFv domain of the fusion retains the affinity and specificity of the parent scFv, while the Fc region is recognized by Protein A, protein G, and anti-Fc antibodies, and is capable of directing antibody-dependent cellular cytotoxicity (ADCC). Yields of the scFv-Fc fusions vary depending on the nature of the scFv, from ~2 mg/l for the best expressor (C25-Fc) to ~300 ug/l for the C6.5-Fc fusion in shaker flasks.

For the proposed *in vivo* anti-metastasis assays of scFv species, a long half-life in the serum will be needed. Previous experiments have shown that the $t_{1/2}$ for the β elimination phase for scFvs are typically 3.5 hours (*9*). In these studies we have demonstrated a $t_{1/2}$ beta 93 hours in a scid mouse for an scFv-Fc fusion, an approximately 25-fold improvement. The terminal half life, however, is still significantly shorter than observed for IgG.

6.3.5 Vector modification to increase scFv-Fc half life

To increase their utility of this system for anti-metastatic scFv characterization, we modified the pPIgG1 vector. As constructed above, cloning scFv genes into this vector requires a PCR step to clone into the alpha factor signal sequence's *Xho*I site and reconstruction of the end of the signal flush with the start of the fusion. Since our N-terminal analysis results show that the final four amino acids (EAEA) are not cleaved from the fusion anyway, the signal sequence was modified to end in EAMA, incorporating a *Nco*I site, for full compatability with the *Nco*I site in the pHEN1 svFv-phage display vector (*13*). This vector was constructed (pPIgG2) and scFv-Fc fusions (C6.5 and C25 expressed at simliar yields as described above. N-terminal protein sequencing of the scFv-Fc fusions indicated that the signal sequence was appropriately cleaved.

Glycosylation pattterns on immunoglobulin species can have profound effects on antibody effector function and pharmacokinetics (reviewed in (*14*)). The serum retention observed for the scFv fusions may be affected by the type and extent of N-linked glycosylation. Yeast in general attach high-mannose residues (*15*), although in Pichia pastoris these residues are generally shorter than in Sacchromyces ceriviciae (*16*). Terminally mannosylated carbohydrates on immunoglobulins can be rapidly cleared by binding to high-affinity mannose receptors in the liver; this clearance can be partially prevented by co-injection of mannan (*17*). Since complete deglycosylation of an IgG can still yield a species with normal antigen affinity and retention of some but not all effector functions (*14, 18-20*), we constructed a further refinement to these vectors (pPIgG3) in which the N-linked glycosylation site from the Fc moiety was deleted. Despite successful removal of the glycosylation site, there was no difference in the serum half life of this fusion compared to wild-type with the glycosylation site.

Dr Powers cloned the anti-uPA-R scFv as scFv-Fc fusions but left his post-doc 6 months early to take a job in the biotechnology industry before the fusions could be evaluated.

7. Key research accomplishments

- Successful generation and characterization of scFv antibodies to uPA and uPA-R.
- Successful characterization of 6 uPA-R scFv and identification of scFv that inhibit uPA binding to uPA-R.
- Generation of a yeast based expression system which permits facile production of scFv-Fc fusions which provide increased stability and a significantly increased serum half life.
- Modification of said expression system to permit simple subcloning of scFv from phage display vectors directly into scFv-Fc expression vectors.

8. Reportable outcomes

8.1 Powers DB, Amersdorfer P, Poul M-A, Shalaby MR and Marks JD. Expression and characterization of single-chain Fv-Fc fusions in Pichia pastoris. J. Immunol. Meth. 251:123-125, 2001.

8.2 Generation of a panel of human uPA and uPA-R scFv antibodies.

8.3 Generation of an expression system for converting scFv into scFv-Fc fusion proteins which have a significantly prolonged serum half life and other Fc mediated functions

8.4 Dr Powers (the post-doctoral fellow) took a job heading a antibody engineering/phage display position as a scientist at a Bay Area company developing antibody based anti-cancer therapeutics.

9. Conclusions

9.1 A panel of human uPA and uPA-R scFv antibodies was generated and characterized.

9.2 scFv antibodies were identified which bl;ock the binding of uPA to uPA-R which may have anti-metastatic activity.

9.3 An expression system was developed for expression of scFv as Fc fusions in yeast. This expression system imparts a significantly longer half life to scFv antibodies, permitting the in vivo evaluation of biologic activity.

9.4 Generation of scFv-Fc fusions also imparts other Fc mediated functions which are critical for in vitro evaluation of antibody activity, such as ADCC.

10. References

1. Sheets, M. D., Amersdorfer, P., Finnern, R., Sargent, P., Lindqvist, E., Schier, R., Hemingsen, G., Wong, C., Gerhart, J. C., and Marks, J. D. (1998) *Proc. Natl. Acad. Sci. USA* Efficient construction of a large non-immune phage antibody library: the production of high-affinity human single-chain antibodies to protein antigens. **95**, 6157-6162.
2. Marks, J. D., Hoogenboom, H. R., Bonnert, T. P., McCafferty, J., Griffiths, A. D., and Winter, G. (1991) *J. Mol. Biol.* By-passing immunization: human antibodies from V-gene libraries displayed on phage. **222**, 581-597.
3. Hoogenboom, H. R. (1997) *Nature Biotechnology* Mix and match: building manifold binding sites. **15**, 125-126.
4. Huston, J. S., Levinson, D., Mudgett-Hunter, M., Tai, M.-S., Novotny, J., Margolies, M. N., Ridge, R. J., Bruccoleri, R. E., Haber, E., Crea, R., and Oppermann, H. (1988) *Proc. Natl. Acad. Sci. USA* Protein engineering of antibody binding sites: recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in *Escherichia coli*. **85**, 5879.
5. McCaffertry, J., Griffiths, A. D., Winter, G., and Chiswell, D. J. (1990) *Nature* Phage antibodies: filamentous phage displaying antibody variable domains. **348**.
6. Adams, G. P., Schier, R., Marshall, K., Wolf, E. J., McCall, A. M., Marks, J. D., and Weiner, L. M. (1998) *Cancer Research* Increased affinity leads to improved selective tumor delivery of single-chain Fv antibodies. **58**, 485-490.
7. Yokata, T., Milenic, D. E., Whitlow, M., and Schlom, J. (1992) *Cancer Research* Rapid tumor penetration of a single-chain Fv and comparison with other immunoglobulin forms. **52**, 3402.
8. Marasco, W. A. (1997) *Gene Therapy* Intrabodies: turning the humoral immune system outside in for intracellular immunization. **4**, 11-15.
9. Huston, J. S., George, A. J. T., Adams, G. P., Stafford, W. F., Jamar, F., Tai, M.-S., McCartney, J. E., Opperman, H., Heelan, B. T., Peters, A. M., Houston, L. L., Bookman, M. A., Wolf, E. J., and Weiner, L. M. (1996) *Q. J. Nucl. Med.* Single-chain Fv radioimmunotargeting. **40**, 320-333.
10. Persic, L., Roberts, A., Wilton, J., Cattaneo, A., Bradbury, A., and Hoogenboom, H. R. (1997) *Gene* An integrated vector system for the eukaryotic expression of antibodies and their fragments after selection from phage display libraries. **187**, 9-18.
11. Amersdorfer, P., C., W., Chen, C., Smith, T., Deshpande, S., Sheridan, R., Finnern, R., and Marks, J. D. (1997) *Infection and Immunity* Molecular Characterization of Murine Humoral Immune Response to Botulinum Neurotoxin Type A Binding Domain as Assessed by Using Phage Antibody Libraries. **65**, 3743-3752.
12. Schier, R., Marks, J. D., Wolf, E. J., Apell, G., Wong, C., McCartney, J. E., Bookman, M. A., Huston, J. S., Houston, L. L., Weiner, L. M., and Adams, G. P. (1995) *Immunotechnology* In vitro and in vivo characterization of a human anti-c-erbB-2 single-chain Fv isolated from a filamentous phage antibody library. **1**, 73-81.
13. Hoogenboom, H. R., Griffiths, A. D., Johnson, K. S., Chiswell, D. J., Hudson, P., and Winter, G. (1991) *Nucleic Acids Research* Multi-subunit proteins on the surface of filamentous phage: methods for displaying antibody (Fab) heavy and light chains. **19**, 4133-4137.
14. Wright, A., and Morrison, S. L. (1997) *Trends in Biotechnology* Effect of glycosylation on antibody function: implications for genetic engineering. **15**, 26-32.

15. Jahn-Schmid, B. (1996) *Immunotechnology* Immunoreactivity of allergen (Bet v 1) conjugated to crystalline bacterial cell surface layers (S-layers). **2**, 103-113.
16. Cregg, J. M., and Higgins, D. R. (1995) *Canadia Journal of Botany* Production of foreign proteins in the yeast *Pichia pastoris.* **73**, S891.
17. Wright, A., and Morrison, S. L. (1994) *J. Exp. Med* Effect of altered CH2-associated carbohydrate structure on the functional properties and in vivo fate of chimeric mouse-human immunoglobulin G1. **180**, 1087-1096.
18. Leatherbarrow, R. (1985) *Mol. Immunol.* Effector functions of a monoclonal aglycosylated mouse IgG2a: binding and activation of complement C1 and interaction with human monocyte Fc receptor. **22**, 407-415.
19. Walker, M. R., Lund, J., Thompson, K. M., and Jeffries, R. (1989) *Biochem. J.* Aglycosylation of human IgG1 and IgG3 monoclonal antibodies can eliminate recognition by human cells expressing Fc gamma R1 and/or Fc gamma RII receptors. **259**, 347.
20. Tao, M.-H., and Morrison, S. L. (1989) *J. Immunol.* Studies of aglycosylated mouse-human IgG. Role of carbohydrate in the structure and effector functions mediated by the human IgG constant region. **143**, 2595-2601.

11. List of personnel receiving support

David Powers, Ph.D.

11. Appendix 1. Tables

ANTIGEN	ELUTED PHAGE TITER		
	round 1	round 2	round 3
urokinase (uPA)	3×10^4	10^7	10^7
urokinase receptor (uPA-R)	5×10^3	5×10^5	2×10^7

Table 1. Titers of eluted phage particles were determined for each round of selection by infecting a culture of E. coli TG1 cells with a dilution of the eluted phage, then plating to determine number of ampicillin resistant colonies.

ANTIGEN	FREQUENCY OF ELISA POSITIVE CLONES		
	round 1	round 2	round 3
urokinase	3/96	12/96	22/96
urokinase receptor	0/96	18/96	28/96

Table 2. 96 clones from each round of selection were assayed by ELISA on the relevant antigen to determine the number of antigen-specific clones. No specific binders were detected for 96 random clones from the unselected library for either antigen (data not shown). A total of 37 total clones were detected for urokinase, and 46 for the urokinase receptor.

		UK displacement*	'epitope'**	expression levels***
1.	G12	-50%	I	1.10 mg
2.	A4	-38%	I	0.172 mg
3.	C2	-47%	I	0.244 mg
4.	F5	-31%	I	0.171 mg
5.	B5	+9%	II	0.921 mg
6.	A9	-29%	I	0.219 mg

Table 3. Expression levels and ability of anti-uPA-R scFv to inhibit binding of uPA to uPA-R

* reduction in ELISA signal in presence of urokinase (UK)

** based on UK displacement

*** mg from a 500 ml prep

ELSEVIER

Journal of Immunological Methods 251 (2001) 123–135

JIM
Journal of Immunological Methods

www.elsevier.nl/locate/jim

Recombinant Technology

Expression of single-chain Fv-Fc fusions in *Pichia pastoris*

David B. Powers[a], Peter Amersdorfer[a,b], Marie-Alix Poul[a,c], Ulrik B. Nielsen[a], M. Refaat Shalaby[d], Gregory P. Adams[e], Louis M. Weiner[e], James D. Marks[a,*]

[a]*Departments of Anesthesia and Pharmaceutical Chemistry, University of California San Francisco, San Francisco, CA 94110, USA*
[b]*Phylos Inc., 128 Spring Street, Lexington, MA 02421, USA*
[c]*UMR CNRS 8532, Laboratoire de Biotechnologies et de Pharmacogénétique Appliquée, Ecole Normale Superieure de Cachan, 61 avenue du President Wilson, 94235 Cachan Cedex, France*
[d]*California Pacific Medical Center, San Francisco, CA 94115, USA*
[e]*Department of Medical Oncology, Fox Chase Cancer Center, Philadelphia, PA 19111, USA*

Received 22 September 1999; received in revised form 23 August 2000; accepted 23 August 2000

Abstract

Phage display technology makes possible the direct isolation of monovalent single-chain Fv antibody fragments. For many applications, however, it is useful to restore Fc mediated antibody functions such as avidity, effector functions and a prolonged serum half-life. We have constructed vectors for the convenient, rapid expression of a single-chain antibody Fv domain (scFv) fused to the Fc portion of human IgG1 in the methylotrophic yeast *Pichia pastoris*. The scFv-Fc fusion protein is secreted and recovered from the culture medium as a disulfide-linked, glycosylated homodimer. The increased size of the dimer (~106 kDa vs. ~25 kDa for a scFv) results in a prolonged serum half-life in vivo, with $t_{1/2}$ of the beta phase of clearance increasing from 3.5 h for a typical scFv to 93 h for a scFv-Fc fusion in mice. The scFv-Fc fusion is capable of mediating antibody-dependent cellular cytotoxicity against tumor target cells using human peripheral blood mononuclear cells as effectors. Finally, the Fc domain is a convenient, robust affinity handle for purification and immunochemical applications, eliminating the need for proteolytically sensitive epitope and/or affinity tags on the scFv.

Keywords: Pichia pastoris; scFv; Antibody engineering; Phage display; HER2/neu; Botulism

Abbreviations: Fv, antibody variable fragment; scFv, single chain Fv fragment; kDa, kilodaltons; V_H, immunoglobulin heavy chain variable region; V_L, immunoglobulin light chain variable region; C_H2 and C_H3, immunoglobulin heavy chain constant regions 2 and 3; ADCC, antibody-dependent cellular cytotoxicity; PCR, polymerase chain reaction; BoNT, botulinum neurotoxin; BoNT/A, BoNT type A; H_c, BoNT binding domain; ELISA, enzyme linked immunosorbent assay; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; HRP, horse radish peroxidase; AMP, ampicillin; KAN, kanamycin; ZEO, Zeocin; PBS, phosphate buffered saline (25 mM NaH_2PO_4, 125 mM NaCl, pH 7.4); MPBS, 2% milk powder in PBS; FACS, fluorescence activated cell sorting; FITC, fluorescein isothiocyanate; PE, phycoerythrin; PBMC, peripheral blood mononuclear cells; RU, resonance units; ECD, extracellular domain; HBS, hepes buffered saline (10 mM Hepes, 150 mM NaCl, pH 7.4); k_{off}, dissociation rate constant; k_{on}, association rate constant; M_r, molecular weight; RT, room temperature; YNB, yeast nitrogen base (with ammonium sulfate without amino acids); i.v., intravenous; i.p., intraperitoneal.

*Corresponding author. San Francisco General Hospital, Room 3C-38, 1001 Potrero Avenue, San Francisco CA, 94110 USA. Tel.: +1-415-206-3256; fax: +1-415-206-3253.

E-mail address: marksj@anesthesia.ucsf.edu (J.D. Marks).

0022-1759/01/$ – see front matter
PII: S0022-1759(00)00290-8

1. Introduction

The modular nature of antibody molecules allows for an almost unlimited number of domain rearrangements and fusions. Antibody engineering allows the researcher to design and use a variety of binding domains, effector domains, as well as non-antibody fusion partners (Chamow and Ashkenazi, 1999; Hoogenboom, 1997). One of the most useful antibody fragments is the single-chain Fv (scFv) in which the isolated antibody V_H and V_L domains are joined by a short peptide linker (Bird et al., 1988; Huston et al., 1988). This monovalent, minimal binding fragment is favored for antibody phage display techniques and phage antibody library construction (Marks et al., 1991; McCafferty et al., 1990).

Once a scFv has been isolated from a phage display library, the binding domain can be characterized with respect to affinity, epitope, and biological activity, as well as subjected to further affinity maturation. The scFv can be used as is, or engineered into other formats (Fv, Fab, $(Fab')_2$, IgG, or fused to other proteins).

For some applications the scFv itself is the desired format. The small size of the scFv, its rapid clearance from the blood, and tumor penetration properties make it the format of choice for tumor targeting and radioimmunoimaging applications (Adams et al., 1995; Adams et al., 1998; Yokata et al., 1992). The single-chain nature of the scFv is also best-suited for intracellular immunization (intrabody) applications (Marasco, 1997). However for other uses, it would be desirable to transfer the antigen-binding properties of the scFv into a full length IgG, to take advantage of avidity effects, effector functions, and the prolonged serum half-life of an immunoglobulin. Increasing the serum half-life of scFv is particularly important for efficient in vivo antigen neutralization since the $t_{1/2}\beta$ of a typical scFv is only 3.5 h in mice (Huston et al., 1996).

One approach is to directly engineer a phage displayed scFv into a full length IgG and express it in mammalian cells (Persic et al., 1997). However this approach requires separate cloning steps for the V_H and V_L domains, and establishing a stable antibody secreting cell line can be time consuming. An alternate approach would be to engineer the scFv into a more 'IgG-like' structure that can be accomplished in a single cloning step, and that can be expressed at high levels in a eucaryotic microorganism such as yeast. We have constructed a vector to rapidly express an scFv-Fc fusion (wherein the scFv is fused to the hinge, C_H2, and C_H3 domains of human IgG1) in the methylotrophic yeast *Pichia pastoris*. The scFv-Fc fusion can easily be constructed in a single cloning step from a scFv and is secreted at high levels as a glycosylated dimer from *Pichia* cultures. The scFv-Fc fusion retains the affinity and specificity of the parent scFv, combined with the bivalency, prolonged serum half-life, and the Fc-mediated ADCC (antibody-dependent cellular cytotoxicity) of an IgG. These yeast-produced scFv-Fc fusions will be useful to rapidly characterize candidate scFv isolated from phage antibody libraries before converting to a full-length IgG, or as an alternative format in its own right.

2. Materials and methods

2.1. Cells and media

Pichia pastoris strain GS115 was obtained from Invitrogen, San Diego, CA. SKBR-3 and SK-OV-3 cells were obtained from the ATCC (American Type Culture Collection, Rockville, MD). Media used: YPD, yeast extract peptone dextrose medium (1% yeast extract, 2% peptone, 2% dextrose); YPDS, YPD medium plus 1 M sorbitol; BMGY, buffered glycerol complex medium (1% yeast extract, 2% peptone, 100 mM phosphate buffer pH 6.0, 1.34% YNB, 4×10^{-5}% biotin, 1% glycerol); BMMY, buffered methanol complex medium (1% yeast extract, 2% peptone, 100 mM phosphate buffer pH 6.0, 1.34% YNB, 4×10^{-5}% biotin, 0.5% methanol).

2.2. pPIgG1, pPIgG1-C25, and pPIgG1-C6.5 plasmid construction

The Fc fragment (hinge, C_H2 and C_H3) of human IgG1 was amplified from the baculovirus expression plasmid pBHuCγ1 (Poul et al., 1995) using primers 'HulgG*Not*Iback' (5′-AAGGAAAAA*GCGGCC-GC*AGAGCCCAAATCTTGTGACAAA-3′) and 'HuIgG*Xba*Ifor' (5′-ACGC*TCTAGA*TCATTTACC-

CGGAGACAGGGAC-3′), which append *Not*I and *Xba*I sites (underlined) onto the 5′ and 3′ ends of the PCR fragment, respectively. The PCR fragment was digested with *Not*I and *Xba*I and subcloned into *Not*I-*Xba*I digested pPICZαA (Invitrogen) to generate plasmid pPIgG1 (for 'Pichia IgG plasmid 1').

C25, a scFv which binds the botulinum neurotoxin type A binding domain (BoNT/A Hc) (Amersdorfer et al., 1997) was PCR amplified from vector pUC119MycHis-C25 using primers 'C25PichiaBack' (5′-CGGCAG*CTCGAG*AAAAGAGAGGCTGAAG-CTCAGGTCCAGCTGCAGGAGTCTGGG-3′) and 'LMB2' (5′-GTAAAACGACGGCCAGT-3′). The 5′ PCR primer C25PichiaBack appends a *Xho*I site (underlined) onto the 5′ end of the scFv for cloning into the *Xho*I site in the leader sequence of plasmid pPIgG1, and also recreates the last few amino acids of the leader sequence fused with the first codon of the C25 scFv. The 3′ PCR primer LMB2 anneals outside of a unique *Not*I site at the end of the C25 scFv, derived from plasmid pHEN1 (Hoogenboom et al., 1991). The PCR fragment was then digested with *Xho*I and *Not*I and subcloned into *Xho*I and *Not*I digested plasmid pPIgG1 generating plasmid pPIgG1-C25. Similarly, C6.5, a scFv which binds the extracellular domain of the HER2/*neu* oncoprotein (Schier et al., 1995) was subcloned into pPIgG1 using primers 'C6.5PichiaBack' (5′-CGGCAG*CKC-GAG*AAAAGAGAGGCTGAAGCTGGCCAGGTG-CAGCTGGTGCAG-3′) and 'LMB2' to create plasmid pPIgG1-C6.5. Following construction, the coding sequences for the Fc region and both the scFvs were sequenced to exclude any PCR-induced errors.

2.3. Electroporation

Pichia pastoris strain GS115 was transformed by electroporation. 10 μg of plasmid was linearized with *Pme*I, phenol-chloroform extracted, ethanol precipitated, and dissolved in 10 μl of dH_2O. Preparation of electrocompetent *Pichia* strain GS115 was done as per supplier's instructions (Invitrogen). 80 μl of electrocompetent cells were mixed with 5-10 μg of linearized plasmid in a 0.2 cm electroporation cuvette, incubated on ice 5 min, and electroporated in a Biorad GenePulser with settings of 1500 V, 25 uF capacitance, and 400 ohms resistance. After pulsing, 1.0 ml of ice cold 1 M sorbitol was added immediately to the cuvette, and the cells transferred to a sterile 15 ml culture tube. The tube was incubated at 30°C without shaking for 1 h, then 1.0 ml YPD medium was added to the tube, and the cells were allowed to recover for 2 h at 30°C at 250 RPM. Transformants were plated (200 μl) on YPDS plates containing 100 μg/ml Zeocin and grown at 30°C to isolate Zeocin-resistant transformants.

2.4. Small scale expression and screening of Pichia transformants

Zeocin-resistant transformants of pPIgG1-C25, pPIgG1-C6.5 and pPICZαA (control) plasmids were grown overnight in BMGY medium at 30°C and 250 rpm shaking in 100 ml glass culture tubes. The next day, the cells were recovered by centrifugation and resuspended to an OD_{600} of 1.0 in BMMY media to induce, and grown again at 30°C and 250 rpm. Fresh methanol was added to a total of 0.5% to maintain induction at 24, 48 and 72 h post induction. After 72 h, samples of the media were analyzed by SDS–PAGE followed by silver stain (Biorad Silver Stain Plus, Biorad, Hercules, CA) and Western blot with polyclonal goat anti-human IgG (Fc-specific) conjugated to horse radish peroxidase (A-0170, Sigma, St. Louis, MO). Detection was with the chemiluminescent substrate ECL (Amersham Pharmacia Biotech, Piscataway, New Jersey).

2.5. Large scale expression and purification of scFv-Fc fusions

250 ml cultures of GS115/pPIgG1-C25 and GS115/pPIgG1-C6.5 were grown overnight in BMGY plus Zeocin (50 μg/ml) and kanamycin (50 μg/ml) until the OD_{600} was 4–6. The cells were recovered by centrifugation, then diluted to an OD_{600} of 1.0 in 1000 ml of fresh BMMY media containing 50 μg/ml kanamycin to induce. The 1000 ml of resuspended cells were divided equally between 4×2 l baffle-bottom flasks to ensure adequate aeration, and grown at 30°C and 250 rpm. Fresh methanol was added to 0.5% to maintain induction at 24, 48, and 72 h. After 72 h, the cells were removed by centrifugation and the scFv-Fc fusion protein purified from the supernatant. Twenty five milliliters of a protease inhibitor cocktail (P2714, Sigma) was added

to reduce proteolysis, and the pH of the supernatant was adjusted to 8.0 by the addition of 1/10 volume of 1.0 M Tris pH 8.0. Proteins were precipitated by the addition of 400 g ammonium sulfate in the cold with constant stirring over a period of 2 h. The precipitate was recovered by centrifugation, dissolved in 40 ml of 25 mM Tris pH 8.0, and dialyzed overnight against two changes of 4 l apiece of 25 mM Tris pH 8.0 at 4°C. After dialysis, the sample was applied to a 1.5 ml Protein G column (Sigma) that had been previously equilibrated with 25 mM Tris pH 8.0. The column was washed once with 10 ml of 100 mM Tris pH 8.0, once with 10 ml of 10 mM Tris pH 8.0, and eluted with 20 ml of 100 mM glycine pH 3.0. 1.0 ml fractions were collected in Eppendorf tubes containing 100 μl of 1.0 M Tris pH 8.0 to neutralize. Peak fractions were determined by absorbance at 280 nm, pooled, concentrated to 0.5 ml on a Centricon 10 concentrator (Amicon, Beverley, MA) and finally gel filtered on a Superdex S-200 column (Amersham Pharmacia Biotech) at a flow rate of 0.5 ml per min in PBS. Protein concentrations were determined based on absorbance at 280 nM using molar extinction coefficients calculated by the method of Gill and von Hippel (Gill and Hippel, 1989). We derived a factor of 1.0 A_{280} is equal to 540 μg/ml for C6.5-Fc and 620 μg/ml for C25-Fc, based on a calculated molecular weight of 106,000 for each.

2.6. Glycosylation analysis

Samples of the scFv-Fc fusions were de-glycosylated using PNGase F (New England Biolabs, Beverley, MA) under denaturing according to the manufacturer's instructions and analyzed by SDS–PAGE followed by silver staining (Biorad Silver Stain Plus).

2.7. Cytotoxicity assay

Procedures were performed in round-bottom microtiter plates (Costar) using a lactate dehydrogenase (LDH) detection kit (Boehringer Manheim). Briefly, 10,000 HER2/*neu* expressing target cells (SKBR-3) in 50 μl of complete media/well were co-cultured with various numbers of effector peripheral blood mononuclear cells (PBMC) added in 100 μl medium/well. Different concentrations of C6.5-Fc fusion protein antibody were added to appropriate cultures in 50 μl volumes (final volume per well, 200 μl). Cultures were performed in triplicates and plates were incubated for 4 h, after which time the plates were centrifuged and 100 μl supernatant were carefully removed from each well and transferred to corresponding wells of a flat bottom microtiter plate. To determine the LDH activity, 100 μl reaction mixture (cytotoxicity kit) are added to each well and the plates were further incubated for 30 min at room temperature for color development. The amount of LDH activity released and detected colorimetrically in the culture supernatant correlates to the proportion of lysed cells. The absorbance of the supernatants is measured at 490 nm and the data are expressed as mean absorbance. The percent cytotoxicity was calculated as follows: percent cytotoxicity $=[(A-B)/(C-B)]\times 100$ where A = the mean absorbance of supernatants from test cultures; B = the mean absorbance of supernatants from cultures containing target cells only (spontaneous LDH release); and C = the mean absorbance of supernatants from cultures containing target cells cultured in the presence of 2% Triton (maximum LDH release). The data were adjusted in accordance with absorbance of supernatants from cultures of PBMC plus C6.5-Fc fusion protein as well as the absorbance of medium devoid of any cells.

2.8. Equilibrium constant (K_d) determination

Affinity measurements were performed on a Kinexa instrument from Sapidyne Instruments, Boise, ID. PMMA beads (Sapidyne Instruments) were coated with antigen (either recombinant BotNT/A Hc fragment or HER2/*neu* extracellular domain) at 50 μg/ml in PBS overnight at 4°C. The beads were washed three times with PBS and blocked with 0.2 μm filtered 2% skimmed milk powder in PBS (MPBS) for 30 min. scFv-Fc fusions were incubated with dilutions of antigen for 4 h in MPBS. The concentration of the C6.5-Fc fusion in the reaction was 10 nM and 1 nM for the C25-Fc fusion. The amount of uncomplexed antibody in the equilibrium reaction was quantified by capture on the antigen coated PMMA beads, followed by detection with 1:1000 dilution of Cy5 labeled goat anti-human Fc antibody (Jackson Laboratories). Reactions were run in duplicate and fluorescent signals were plotted

as function of antigen concentration. Equilibrium constants were determined using the software provided by the manufacturer (Sapidyne Instruments). The Kinexa assay has been described in detail (Blake et al., 1997).

2.9. Radiolabeling

The C6.5-Fc was labeled with iodine-125 using iodobeads (# 28665X, Pierce; Rockford IL) according to the methods described by the manufacturer. Briefly, 0.5 mg of C6.5-Fc in 250 ml 0.1 M phosphate buffer (pH 6.5) were combined with 0.5 mCi (1.25 ml) of iodine-125 (#NEZ033H, DuPont NEN, Wilmington, DE), and one iodobead. The mixture was incubated for 3 min at room temperature. Unincorporated radioiodine was separated from the labeled protein by gel filtration using the G-50-80 centrifuged-column method (Meares et al., 1984). The immunoreactivity of the C6.5-Fc was evaluated in a live cell binding assay utilizing HER2/*neu* expressing SK-OV-3 cells (Adams et al., 1993). Ten nanograms of labeled C6.5-Fc in 100 ml PBS was added in triplicate to 5×106 SK-OV-3 cells in 15 ml polypropylene centrifuge tubes. After a 30 min incubation at room temperature the cells were washed with 2.0 ml of PBS and centrifuged for 5 min at 500×*g*. Supernatants were separated from the cell pellets, both were transferred to 12×75 counting tubes, counted in a gamma well counter (Gamma 4000, Beckman Instruments, Irvine, CA) and the percentage of activity associated with the cell pellet was determined. Fifty three percent of the activity was found to be associated with the cell pellet. While the maximum possible value in this assay is about 80%, the degree of retention is dictated by a number of factors including the affinity of the molecule for the target antigen. These results are within the normal range observed for radioiodinated C6.5 scFv, indicating to us that the C6.5-Fc was still reactive with cell surface HER2/*neu*.

2.10. Pharmacokinetics

Four-month-old inbred male C.B17/Icr-*scid* (scid) mice were obtained from the Fox Chase Cancer Center Laboratory Animal Facility. Lugol's solution was placed in the drinking water to block thyroid accumulation of radioiodine. Two days later, pharmacokinetic studies were initiated. Twenty micrograms of ^{125}I-C6.5-Fc were administered to three cohorts of four mice by i.v. tail vein injection and to three cohorts of four mice by i.p. injection. Total injected doses were determined by counting each animal on a Series 30 multichannel analyzer/probe system (probe model #2007, Canaberra, Meridian, CT). Blood samples (20-75 μl) were obtained by retro-orbital bleeds from all mice at 5 min post injection and then from alternating groups at subsequent time points (e.g., group I at 15 min, group II at 30 min, group III at 1 h, etc.). The blood samples were counted along with standards in a gamma counter (Beckman, 4000) and the percent of the injected dose retained per ml (% ID/ml) of blood over time was determined for each mouse. The mean values were determined for each time point and the pharmacokinetics were determined using the NCOMP program (Laub and Gallo, 1996).

3. Results

3.1. Plasmid constructions

We have constructed a plasmid for the expression of single-chain antibody Fv domains (scFv) fused to the human IgG1 Fc domain to make a bivalent, ~106,000 molecular weight scFv-Fc fusion (Fig. 1). Plasmid pPIgG1 for the expression of the scFv-Fc fusions (comprising the scFv followed by the hinge, C_H2 and C_H3 domains of human IgG1) is based on the *Pichia pastoris* expression plasmid pPICZαA (Invitrogen). This plasmid uses the alcohol oxidase (AOX1) promoter for high level expression of heterologous proteins, which are secreted to the media under direction of the *Saccharomyces cerevisiae* a-factor signal sequence.

Details of the plasmid and the cloning sites are shown in Fig. 2. The human hinge, C_H2, and C_H3 domains of IgG1 were cloned into the *Not*I and *Xba*I sites of pPICZαA, creating plasmid pPIgG1. A stop codon was included in the construct following the final amino acid of the C_H3 domain; therefore the myc epitope tag and $(His)_6$ sequences from pPICzαA are not expressed in the fusion protein.

scFv genes to be expressed as scFv-Fc fusions in pPIgG1 are amplified by PCR and subcloned into pPIgG1 using the 5′ *Xho*I site in the alpha factor

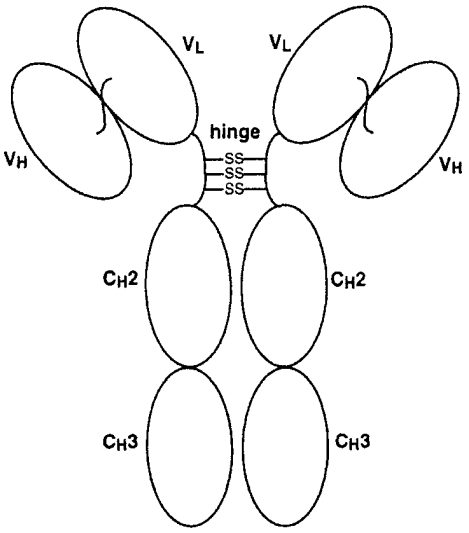

Fig. 1. Schematic representation of a dimeric scFv-Fc fusion. V_H, heavy chain variable domain; V_L, light chain variable domain: C_H2 and C_H3, heavy chain constant domains 2 and 3; –SS– indicates disulfide bonds between the hinge domains.

signal sequence and the 3′ *Not*I site which sits just before the beginning of the IgG hinge (Fig. 2). The 5′ PCR primer, in addition to appending an *Xho*I site, must also be designed to recreate the amino acid sequence of the alpha amylase signal from the *Xho*I site to the end of the signal peptide, positioning the 5′ end of the scFv flush with the end of the signal. The 3′ PCR primer can be either a sequence-specific primer which appends a *Not*I site onto the end of the scFv gene, or a universal downstream primer for amplifying from vectors such as the phage display vector pHEN1 which already has an in-frame *Not*I site positioned at the end of the scFv gene (Hoogenboom et al., 1991). We subcloned two model scFvs into the pPIgG1 vector for evaluation: C25, which recognizes the botulinum neurotoxin serotype A binding domain (BotNT/A Hc) (Amersdorfer et al., 1997), and C6.5, which recognizes the HER2/*neu* protein (Schier et al., 1995). The constructs were electroporated into *Pichia pastoris* strain GS115 for expression.

3.2. Small scale expression and screening of *Pichia* transformants

Since protein expression levels in *Pichia* from integrated pPICZα-derived plasmids can vary depending on site of integration and copy number, we

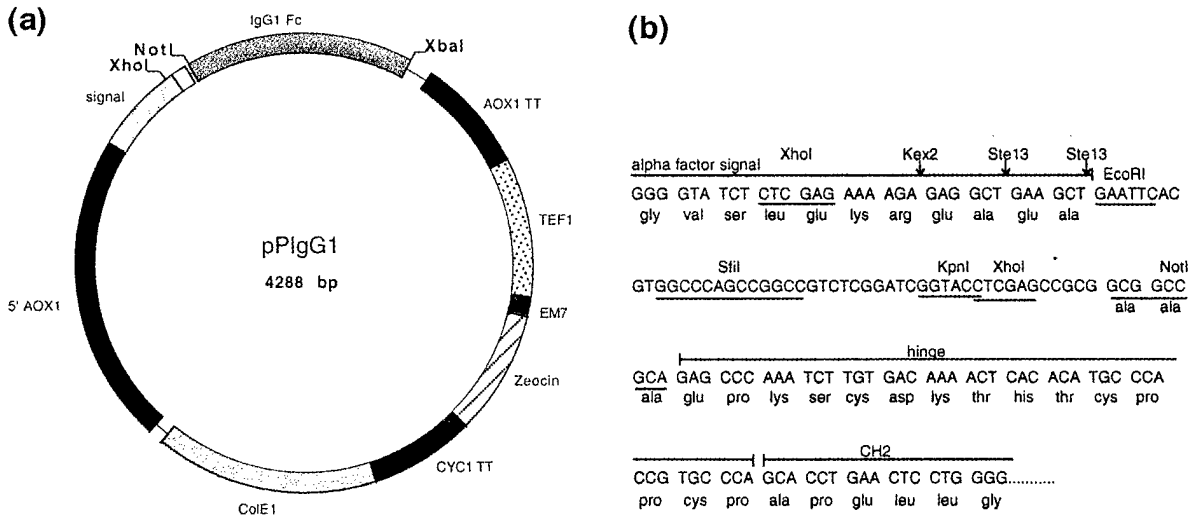

Fig. 2. (A) Schematic of plasmid pPIgG1. 5′ AOX1, alcohol oxidase 1 promoter; AOX1 TT, transcriptional terminator from *Pichia pastoris* AOX1 gene; TEF1 promoter, transcriptional elongation factor 1 promoter from *Saccharomyces cerevisiae*; EM7 promoter, synthetic prokaryotic promoter; Zeocin, Zeocin resistance gene; CYC1 TT, transcriptional terminator from *Saccharomyces cerevisiae* CYC1 gene; ColE1, ColE1 origin of replication. (B) Details of the cloning sites of pPIgG1. Restriction endonuclease sites are underlined, arrows indicate cleavage sites within the alpha mating factor signal sequence for Kex2 and Ste13 proteases.

screened multiple transformants for scFv-Fc expression levels. Four clones apiece of the pPIgG1-C25 and pPIgG1-C6.5 Zeocin-resistant transformants (along with pPICZαA as a control) were grown and induced in 10 ml of BMMY media at 30°C for 72 h as described in Materials and methods. Cultures supernatants were analyzed on SDS–PAGE under reducing conditions followed by silver stain detection. Results show that a new band appears in the media with an apparent molecular weight of ~55,000 daltons, in good agreement with the predicted 53,000 dalton molecular weight of a reduced scFv-Fc fusion (Fig. 3 panel A). Close inspection shows that this band is actually a closely spaced doublet of bands with similar mobilities. Western blotting analysis demonstrates that these bands react with an anti-human Fc specific antibody (Fig. 3 panel B). No anti-Fc reactive species were seen in *Pichia* GS115 cells transfected with the control plasmid (pPICZαA). On non-reducing SDS–PAGE gels, the scFv-Fc protein bands have approximately twice the apparent molecular weight, indicating that the scFv-Fc fusions are secreted to the media primarily as disulfide-linked dimers (not shown). Expression levels of the C6.5-Fc clones are somewhat less than that of the C25-Fc clones. Two well-expressing clones were chosen for large scale expression and analysis.

3.3. Large scale expression and purification of scFv-Fc fusions

One liter of culture media was used to generate pure C25-Fc and C6.5-Fc fusions for characterization. Briefly, the purification employed was ammonium sulfate precipitation of the proteins from the culture supernatants, followed by dialysis, Protein G affinity chromatography, and gel filtration chroma-

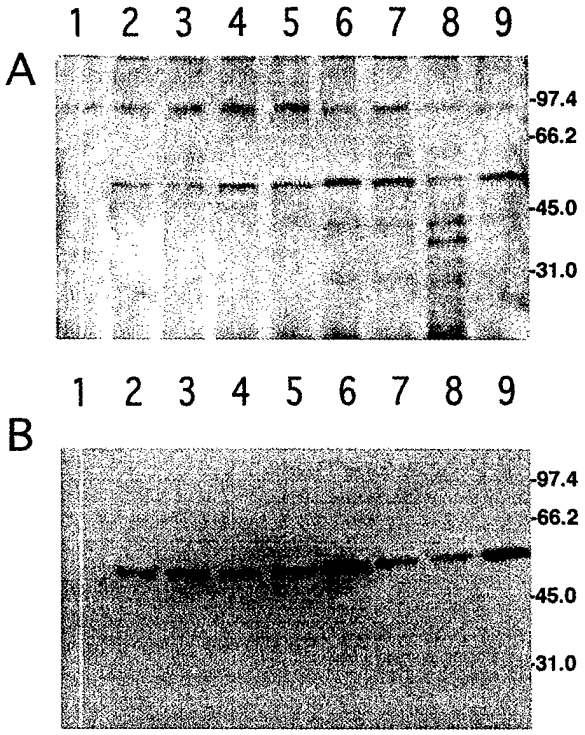

Fig. 3. Analysis of scFv-Fc expression in *Pichia pastoris* strain GS115. (A) Silver stained SDS–PAGE gel of culture supernatants. Lane 1, pPICZαA control transformant; lanes 2-5, pPIgG1-C6.5 transformants; lanes 6-9, pPIgG1-C25 transformants. (B) Western blot of a duplicate of the above gel using anti-human IgG (Fc-specific)-HRP (horse radish peroxidase) conjugate, followed by ECL development. Molecular weight markers (kDa) are indicated on right of each gel.

tography. Fig. 4, panel A shows fractions from a typical purification. After ammonium sulfate precipitation, the ~55 kDa scFv-Fc fusion protein doublet is clearly visible (lanes 1 and 4). After Protein G affinity chromatography (lanes 2 and 5) the scFv-Fc fusion protein is substantially pure, with a number of smaller bands copurifying which may represent breakdown products which also bind to Protein G. These lower molecular weight species are removed by gel filtration on Superdex S-200 (Amersham Pharmacia Biotech) (lanes 3 and 6). The yield after purification was ~2 mg/l of culture for C25-Fc and ~300 μg/l of culture for the C6.5-Fc fusion. The purified scFv-Fc fusion protein is a disulfide-linked dimer, as shown by SDS–PAGE analysis under reducing and non-reducing conditions (Fig. 4B). The dimeric nature of the scFv-Fc was also confirmed by molecular weight analysis on Superdex S-200 gel filtration vs. molecular weight standards (not shown).

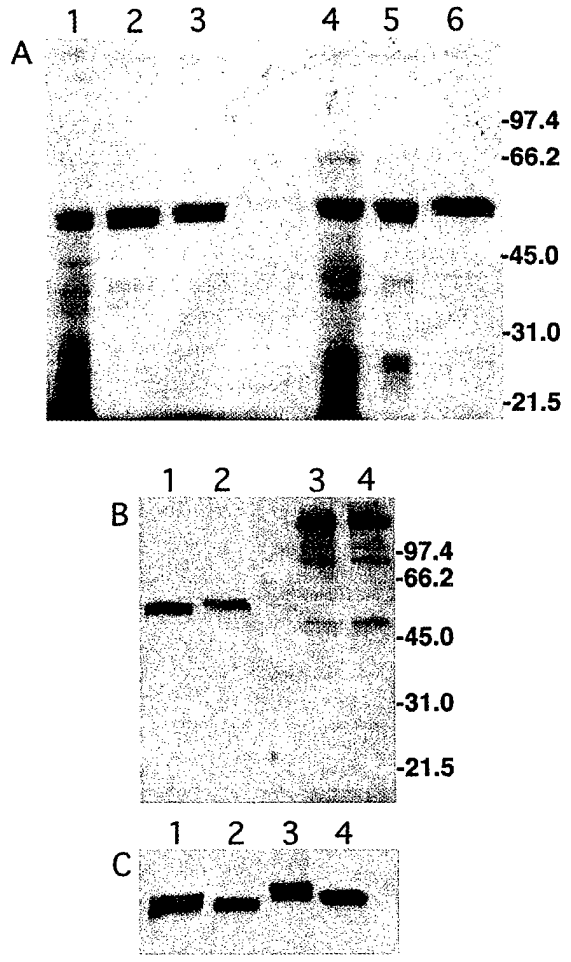

Fig. 4. (A) SDS–PAGE analysis of samples from representative purifications of the C6.5 and C25-Fc fusion proteins. Lane 1, ammonium sulfate pellet of C6.5-Fc; lane 2, protein G pool of C6.5-Fc; lane 3, S200 pool of C6.5-Fc; lane 4, ammonium sulfate pellet of C25-Fc; lane 5, protein G pool of C25-Fc; lane 6, S200 pool of C25-Fc. (B)SDS–PAGE analysis of the purified scFv-Fc fusions under reducing and non-reducing conditions. Lanes 1 and 3, purified C6.5-Fc, lanes 2 and 4, purified C25-Fc protein. Lanes 1 and 2 are under reducing conditions, lanes 3 and 4 under non-reducing conditions. Molecular weight standards (kDa) are to the right. (C) PNGase F analysis of glycosylation of the C6.5-Fc and C25-Fc fusions. Lane 1 and 2, C6.5-Fc; lanes 3 and 4, C25-Fc. Lanes 1 and 3 are minus PNGase F; lanes 2 and 4 are plus PNGase F.

The final purified scFv-Fc proteins migrate in reducing SDS–PAGE as a tight doublet of bands with an apparent molecular weight of ~55,000 daltons. The molecular weight heterogeneity observed could be due to differences in glycosylation, or in the processing of the alpha amylase signal peptide. To explore these possibilities, the purified fusions were subjected to N-terminal sequencing and de-glycosylation analysis with PNGase F.

N-terminal analysis results indicated that both the upper and lower bands of the doublet, for both C6.5-Fc and C25-Fc proteins, begin with the amino acid sequence glu-ala-glu-ala..., indicating that signal peptide cleavage had occurred after the Kex2 site in the alpha amylase leader, but that Ste13 cleavage had not occurred (Fig. 2). The samples were further analyzed by deglycosylation with PNGase F (Fig. 4C). After deglycosylation of both the fusion proteins, the doublet collapsed to a single band, indicating that the observed heterogeneity is due to N-linked glycosylation of the higher molecular band and no glycosylation of the lower molecular weight band.

3.4. Affinity (K_d) analysis

We wished to show that the scFv antigen binding site in the scFv-Fc fusion retained the affinity of the unfused scFv. The affinities of our model scFvs for their antigens have been previously determined by kinetic analysis of association and dissociation rate constants (k_{on} and k_{off}) by surface plasmon resonance (Amersdorfer et al., 1997; Schier et al., 1995);

from the k_{on} and k_{off} values the equilibrium dissociation constants (K_ds) of C6.5 and C25 were calculated to be 16 nM and 1 nM, respectively. We determined equilibrium dissociation constants under equilibrium conditions using a Kinexa instrument (Sapidine Instruments) (Blake et al., 1997). We determined the K_ds of the C6.5-Fc and C25-Fc fusion proteins to be 4.6 nM and 0.5 nM, respectively, for their cognate antigens. While the different experimental techniques do not allow a direct comparison of the K_d values, and we cannot rule out subtle differences in affinity between the scFv and scFv-Fc fusion formats, it is clear that the scFv binding sites retain high affinity for their cognate antigens in the scFv-Fc fusion format.

3.5. ADCC assays

The C6.5-Fc fusion protein was assayed to determine if the Fc domain of the fusion was functionally able to direct antibody-dependent cell cytotoxicity (ADCC) towards antigen expressing target cells. 10,000 HER2/*neu* expressing SKBR3 target cells were co-cultured with effector PBMC (peripheral blood mononuclear cells) at 100:1, 50:1, and 25:1 ratios in the presence of various concentrations of C6.5-Fc. After 4 h incubation, the samples were analyzed for percent cell lysis by lactate dehydrogenase (LDH) release. Results (Fig. 5) show PBMC killing of target cells occurs in a dose dependent fashion with C6.5-Fc.

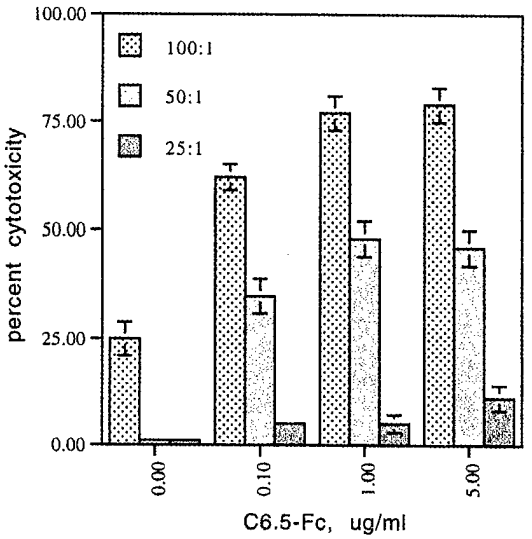

Fig. 5. ADCC (antibody-dependent cellular cytotoxicity) assays of the C6.5-Fc fusion protein using PBMC effector cells and HER2/*neu* expressing SKBR3 cells as target. Shaded bars indicates different effector cell: target cell ratios.

3.6. Pharmacokinetics

Single-chain Fv antibodies are rapidly cleared from the bloodstream in mouse models, with typical $t_{1/2}$ of the beta clearance of approximately 3.5 h (Huston et al., 1996). Similarly, in previous studies of C6.5 scFv pharmacokinetics in scid mice, a $t_{1/2}$ β of 3.0 h was determined (G. Adams, unpublished data). This rapid clearance prevents the characterization of scFv in animal models for efficacy where a longer serum half-life is required to observe a biological effect. We wished to determine if the fusion of a model single-chain antibody Fv domain to a Fc domain would impart a longer serum half life to the scFv. C6.5-Fc was labeled with iodine-125 using iodobeads, evaluated in a live cell binding assay utilizing HER2/*neu* expressing SK-OV-3 cells for retention of activity. (Adams et al., 1993), and administered to four month old inbred male C.B17/Icr-*scid* (scid) mice as described in Materials and methods. Both intravenous (tail vein) and intraperitoneal routes of administration were studied. Results are shown in Fig. 6. After a rapid tissue distribution (alpha phase), the scFv-Fc fusion is cleared in the beta phase of elimination more slowly, with the $t_{1/2}$ β of 37.3 h for intravenous and 92.8 h for intraperitoneal injections. This represents a more than 12-fold and more than 30-fold improvement over the 3.0 h $t_{1/2}$ β observed for a C6.5 scFv alone. In contrast to a typical scFv, which is practically undetectable in mouse serum 12 h past injection, the slower clearance of the scFv-Fc fusion results in substantial levels of scFv in the blood at longer times, with approximately 5% of the injected dose still in the serum at 48 h after injection for both i.v. and i.p. injections.

4. Discussion

The single-polypeptide, minimally sized scFv antibody fragment is useful for the design and

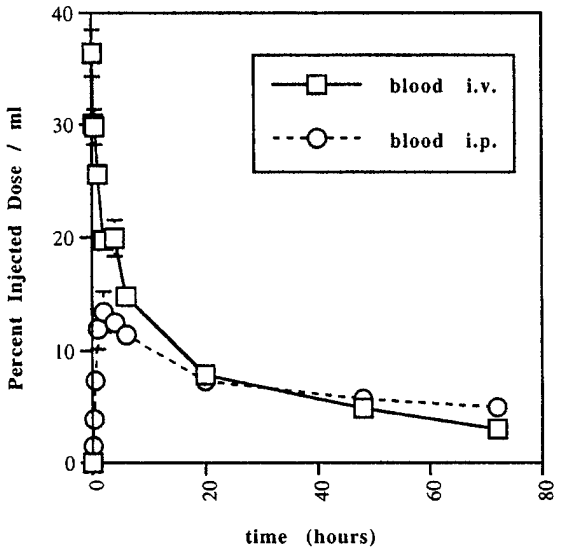

Fig. 6. Kinetics of clearance of C6.5-Fc fusion from mouse serum. Mice received ^{125}I-labeled C6.5-Fc protein by either intravenous or intraperitoneal injection. Each data point represents the mean value for four mice.

construction of phage display antibody libraries, as fusion proteins, and as intrabodies; in addition the small size is best suited to tumor targeting due to superior tissue penetration (Yokata et al., 1992). However useful, the scFv format suffers from a number of key limitations. The lack of avidity due to monovalent binding can limit the effectiveness and/or sensitivity of scFv fragments in many immunochemical applications such as FACS and ELISA. Also, the scFv fragment typically needs to be 'tagged' in some way, such as a hexahistidine or epitope tag, to allow detection and purification. Perhaps most importantly, in vivo characterization of scFv in animal models can be impossible because of their rapid clearance from the bloodstream due to their small size. (Huston et al., 1996)

It would be desirable to be able to easily reengineer a scFv into an IgG-like format that combines the affinity and specificity of the scFv with the bivalency, pharmacokinetics, and effector functions of a complete immunoglobulin. One way is to clone the V_H and V_L genes from the scFv and reclone them into a full-length IgG expressing vector (Persic et al., 1997). However this requires separate cloning steps for the V_H and V_L, and it can require significant amounts of time to make a stable IgG secreting tissue culture line.

As an alternative, we have constructed a vector, pPIgG1, to express a scFv-Fc fusion (in which the scFv is fused to the hinge, C_H2, and C_H3 domains of human IgG1) in the yeast *Pichia pastoris* (Fig. 1). The scFv is expressed and secreted as a glycosylated, disulfide-linked dimer at high yields in the culture medium. The scFv-Fc fusion combines the affinity and specificity of the scFv antigen binding site with Fc-mediated dimerization. In addition, the Fc domain is a convenient affinity 'handle' for the purification and detection of the scFv species by reagents like protein A, protein G, and anti-Fc antibodies (Figs. 3B and 4A). An additional major advantage of *Pichia pastoris* over mammalian cell culture production is that *Pichia* can be readily fermented to high OD and high protein expression levels (Cregg and Higgins, 1995). This construct is similar to scFv-Fc fusions that other workers have made in mammalian and insect cells (Brocks et al., 1997; Hayden et al., 1994; Kato et al., 1995; Ma et al., 1996; Shu et al., 1993), however this is the first time to our knowledge that such a fusion has been expressed in yeast.

We have cloned and expressed two model scFv into pPIgGl for evaluation of our system: C25, which recognizes the botulinum neurotoxin serotype A binding domain (BotNT/A Hc) (Amersdorfer et al., 1997), and C6.5, which recognizes the HER2/*neu* protein (Schier et al., 1995). Yields of the scFv-Fc fusions vary depending on the nature of the scFv, from ~2 mg/l for the best expressor (C25-Fc) to ~300 μg/l for the C6.5-Fc fusion in shaker flasks. By an equilibrium K_d determination, we have shown that the scFv domain of the fusion retains the affinity of the parent scFv for its cognate antigen.

The use of scFv antibody fragments in FACS can be limited due to monovalent binding and lack of a good detection method. In favorable circumstances, we and other investigators have demonstrated the use of scFv from phage display libraries as FACS reagents, either directly as scFv-phage, or as isolated scFv fragments (de Kruif et al., 1995). However, we have found that the utility of scFv as FACS reagents must be evaluated on a case-by-case basis. For example, previous studies of C6.5 scFv protein on HER2/*neu* positive SK-OV-3 cells showed that the

half-life ($t_{1/2}$) of the monovalent C6.5 scFv on the cell surface was much less than 5 min; efficient staining required preparing biotinylated scFv, staining the cells with biotinylated scFv, fixing the stained cells in paraformaldehyde, and detection with PE labeled streptavidin (Schier et al., 1996). In contrast, we routinely use the C6.5-Fc fusion protein in FACS assays on HER2/*neu* expressing cells without any modification of the protein or fixing of the target cells; detection is accomplished with fluorescently labeled anti-Fc antibodies (M.A. Poul, not shown). In addition, superior binding of a bivalent scFv-Fc to a cell surface antigen due to avidity also results in superior sensitivity over a monovalent scFv. This could be of obvious benefit for use of phage display library derived scFv to study cell surface expression of target molecules, especially where low levels of cell surface antigen need to be detected.

For in vivo characterization of scFv species, a long serum half-life will be needed in many instances to observe a biological effect. Previous experiments in mice have shown that the $t_{1/2}$ for the beta phase of elimination for scFvs are typically 3.5 h (Huston et al., 1996). Similarly, in previous studies of C6.5 scFv pharmacokinetics in scid mice, a $t_{1/2}$ β of 3.0 h was determined (G. Adams, unpublished data). We have shown in these experiment a significant increase of the $t_{1/2}$ β of the C6.5-Fc fusion to 37.3 and 92.8 h for intravenous and intraperitoneal injections, respectively. This represents greater than 12-fold and 30-fold improvements in the half life, resulting in significant serum levels (~5% of the injected dose/ml) even at 48 h post injection. We have recently used the superior pharmacokinetics of pPIgG1 derived scFv-Fc fusions to demonstrate in vivo Botulinum toxin neutralization with scFv selected from phage display libraries (Amersdorfer, Powers, and Marks, manuscript in preparation).

In contrast to a typical immunoglobulin, pharmacokinetic analysis of the C6.5-Fc fusion when administered by i.v. injection shows a significant and rapid clearance of ~80% of the fusion protein in the alpha phase (Fig. 6). We speculate that this may be due to the nature of carbohydrate attached to the *Pichia* derived scFv-Fc fusion. Glycosylation patterns on immunoglobulin species can have profound effects on antibody effector function and pharmacokinetics (reviewed in Wright and Morrison, 1997). Yeast in general attach high-molecular weight mannose structures (Jahn-Schmid et al., 1996), although in *Pichia pastoris* these residues are generally shorter than in *Saccharomyces cerevisiae* (Cregg and Higgins, 1995). Terminally mannosylated carbohydrates on immunoglobulins can be rapidly cleared by binding to high-affinity mannose receptors in the liver; this clearance can be partially prevented by co-injection of mannan (Wright and Morrison, 1994). Since complete deglycosylation of an IgG results in normal antigen affinity while retaining some (but not all) effector functions (Leatherbarrow et al., 1985; Tao and Morrison, 1989; Walker et al., 1989; Wright and Morrison, 1997), it may be possible to further improve the pharmacokinetics of the *Pichia* derived scFv-Fc fusions by eliminating the N-linked glycosylation site from the Fc domain by site-directed mutagenesis.

We have recently modified the pPIgG1 vector to further increase its utility. At present cloning into pPIgG1 requires using the 5′ *Xho*I site in the alpha factor signal sequence and reconstruction by the PCR primer of the last few amino acids in the signal sequence. Since N-terminal sequence analysis shows that the final four amino acids of the signal (glu-ala-glu-ala . . .) are not cleaved from the C6.5-Fc and C25-Fc fusions, we have mutated the alpha factor signal sequence to end in glu-ala-*met*-ala . . . , and incorporated a *Nco*I site (CCATGG) around the minus 2 position methionine codon (ATG). In addition a second *Nco*I site in the pPICZαA vector was eliminated by site-directed mutagenesis. This modified vector (pPIgG2) allows scFv to be removed and subcloned by a simple *Nco*I-*Not*I digest from our preferred scFv phage display vector pHEN1 (Hoogenboom et al., 1991) (D. Powers, unpublished results).

As more and more scFv from phage display libraries are developed for research, diagnostic, and therapeutic uses, it will be increasingly more important to have simple, rapid techniques to reengineer the scFv into appropriate formats for characterization. The *Pichia pastoris* scFv-Fc fusion protein system allows a simple, rapid way to combine the affinity and specificity of a scFv with the Fc mediated bivalency, prolonged serum half life, and effector functions of an IgG.

Acknowledgements

We would like to thank Mr. Tom Purcell of Invitrogen Co. for helpful discussions, Mr. Steve Chen for technical assistance, and Dr. Chris Turk of UCSF for N-terminal sequence analysis. This work was partially funded by DAMD17-94-C-4034, DAMD17-94-J-4433, and NIH CA65559.

References

Adams, G.P., McCartney, J.E., Tai, M.-S. et al., 1993. Highly specific in vivo tumor targeting by monovalent and divalent forms of 741F8 anti-c-erbB-2 single chain Fv. Cancer Res. 53, 4026.

Adams, G.P., McCartney, J.E., Wolf, E.J. et al., 1995. Enhanced tumor specificity with stably iodinated 741F8-2 (sFv')2, an anti-c-erbB-2 single chain Fv dimer. J. Nucl. Med. 36, 2276.

Adams, G.P., Schier, R., Marshall, K. et al., 1998. Increased affinity leads to improved selective tumor delivery of single-chain Fv antibodies. Cancer Res. 58, 485.

Amersdorfer, P., Wong, C., Chen, C. et al., 1997. Molecular characterization of murine humoral immune response to botulinum neurotoxin type A binding domain as assessed by using phage antibody libraries. Infect. Immun. 65, 3743.

Bird, R.E., Hardman, K.D., Jacobson, J.W. et al., 1988. Single-chain antigen-binding proteins. Science 242, 423.

Blake, D.A., Khosraviani, M., Pavlov, A.R., Blake, R.C., 1997. Characterization of a metal-specific monoclonal antibody. Immunochem. Technol. Environ. Applic. 657, 49.

Brocks, B., Rode, H.J., Klein, M. et al., 1997. A TNF receptor antagonistic scFv, which is not secreted in mammalian cells, is expressed as a soluble mono- and bivalent scFv derivative in insect cells. Immunotechnology 3, 173.

Chamow, S., Ashkenazi, M.A., 1999. Antibody Fusion Proteins. John Wiley, New York.

Cregg, J.M., Higgins, D.R., 1995. Production of foreign proteins in the yeast *Pichia pastoris*. Canadian J. Botany 73, S891.

de Kruif, J., Terstappen, L., Boel, E., Logtenberg, T., 1995. Rapid selection of cell subpopulation-specific human monoclonal antibodies from a synthetic phage antibody library. Immunology 92, 3938.

Gill, S.C., Hippel, P.H.V., 1989. Calculation of protein extinction coefficients from amino acid sequence data. Anal. Biochem. 182, 319.

Hayden, M.S., Linsley, P.S., Gayle, M.A. et al., 1994. Single-chain mono- and bispecific antibody derivatives with novel biological properties and antitumor activity from a COS cell transient expression system. Ther. Immunol. 1, 3.

Hoogenboom, H.R., 1997. Mix and match: building manifold binding sites. Nature Biotechnol. 15, 125.

Hoogenboom, H.R., Griffiths, A.D., Johnson, K.S. et al., 1991. Multi-subunit proteins on the surface of filamentous phage: methods for displaying antibody (Fab) heavy and light chains. Nucleic Acids Res. 19, 4133.

Huston, J.S., George, A.J.T., Adams, G.P. et al., 1996. Single-chain Fv radioimmunotargeting. Q. J. Nucl. Med. 40, 320.

Huston, J.S., Levinson, D., Mudgett-Hunter, M. et al., 1988. Protein engineering of antibody binding sites: recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in Escherichia coli. Proc. Natl. Acad. Sci. USA 85, 5879.

Jahn-Schmid, B., Graninger, M., Glozik, M. et al., 1996. Immunoreactivity of allergen (Bet v 1) conjugated to crystalline bacterial cell surface layers (S-layers). Immunotechnology 2, 103.

Kato, T., Sato, K., Suzuki, S. et al., 1995. Mammalian expression of single chain variable fragments dimerized by Fc regions. Mol. Biol. Rep. 21, 141.

Laub, P.B., Gallo, J.M., 1996. NCOMP, a Windows-based computer program for noncompartmental analysis of pharmacokinetic data. J. Pharm. Sci. 85, 393.

Leatherbarrow, R.J., Rademacher, T.W., Dwek, R.A. et al., 1985. Effector functions of a monoclonal aglycosylated mouse IgG2a: binding and activation of complement component C1 and interaction with human monocyte Fc receptor. Mol. Immunol. 22, 407.

Ma, S., Thompson, J., Hu, H., Neville, D.M., 1996. Expression and characterization of a divalent chimeric anti-human CD3 single-chain antibody. Scand. J. Immunol. 43, 134.

Marasco, W.A., 1997. Intrabodies: turning the humoral immune system outside in for intracellular immunization. Gene Ther. 4, 11.

Marks, J.D., Hoogenboom, H.R., Bonnert, T.P., McCafferty, J., Griffiths, A.D., Winter, G., 1991. By-passing immunization: human antibodies from V-gene libraries displayed on phage. J. Mol. Biol. 222, 581.

McCafferty, J., Griffiths, A.D., Winter, G., Chiswell, D.J., 1990. Phage antibodies: filamentous phage displaying antibody variable domains. Nature 348, 552.

Meares, C.F., McCall, M.J., Reardan, D.T., Goodwin, D.A., Diamanti, P.I., McTigue, M., 1984. Conjunction of antibodies with bifunctional chelating agents: isothiocyanate and bromoacetamide reagents, methods of analysis, and subsequent addition of metal ions. Anal. Biochem. 142, 68.

Persic, L., Roberts, A., Wilton, J., Cattaneo, A., Bradbury, A., Hoogenboom, H.R., 1997. An integrated vector system for the eukaryotic expression of antibodies and their fragments after selection from phage display libraries. Gene 187, 9.

Poul, M.-A., Cerutti, M., Chaabihi, H., Devauchelle, G., Kaczorek, M., Lefranc, M.-P., 1995. Design of cassette baculovirus vectors for the production of therapeutic antibodies in insect cells. Immunotechnology 1, 189.

Schier, R., Marks, J.D., Wolf, E.J. et al., 1995. In vitro and in vivo characterization of a human anti-c-erbB-2 single-chain Fv isolated from a filamentous phage antibody library. Immunotechnology 1, 73.

Schier, R., McCall, A., Adams, G.P. et al., 1996. Isolation of picomolar affinity anti-c-erbB-2 single-chain Fv by molecular evolution of the complementarity determining regions in the center of the antibody binding site. J. Mol. Biol. 263, 551.

Shu, L., Qi, C.F., Schlom, J., Kashmiri, S.V., 1993. Secretion of a single-gene-encoded immunoglobulin from myeloma cells. Proc. Natl. Acad. Sci. USA 90, 7995.

Tao, M.-H., Morrison, S.L., 1989. Studies of aglycosylated chimeric mouse-human IgG. Role of carbohydrate in the structure and effector functions mediated by the human IgG constant region. J. Immunol. 143, 2595.

Walker, M.R., Lund, J., Thompson, K.M., Jefferis, R., 1989. Aglycosylation of human IgG1 and IgG3 monoclonal antibodies can eliminate recognition by human cells expressing Fc gamma RI and/or Fc gamma RII receptors. Biochem. J. 259, 347.

Wright, A., Morrison, S.L., 1994. Effect of altered CH2-associated carbohydrate structure on the functional properties and in vivo fate of chimeric mouse-human immunoglobulin G1. J. Exp. Med 180, 1087.

Wright, A., Morrison, S.L., 1997. Effect of glycosylation on antibody function: implications for genetic engineering. Trends Biotechnol. 15, 26.

Yokata, T., Milenic, D.E., Whitlow, M., Schlom, J., 1992. Rapid tumor penetration of a single-chain Fv and comparison with other immunoglobulin forms. Cancer Res. 52, 3402.